J. Clin. Chem. Clin. Biochem.
Vol. 26, 1988, pp. 635–644

Recommendation for the Measurement of "Alanine Aminopeptidase" in Urine[1])

By *H. Mattenheimer*

Department of Biochemistry and Office of Consolidated Laboratory Services, Rush-Presbyterian-St. Luke's Medical Center, Chicago, USA

W. Frölke

Boehringer-Ingelheim KG, Ingelheim, FRG

H. Grötsch

Hoechst AG, Frankfurt/M, FRG

D. Maruhn

Bayer AG, Wuppertal, FRG

Z. Simane

E. Merck, Darmstadt, FRG

(Received March 1/June 20, 1988)

Summary: A method is recommended for the measurement of the catalytic concentration of alanine aminopeptidase in the urine of man, rat, and dog, using *L*-alanine-4-nitroanilide as substrate. In currently used methods, substrate concentrations between 1.7 and 2.0 mmol/l are used. Kinetic experiments show, however, that the reaction is inhibited by substrate concentrations exceeding 0.8 (man), 0.3 (rat) and 0.5 mmol/l (dog); these concentrations lie in the range of the K_m values. Assay conditions were therefore chosen to give the lowest possible K_m. The K_m value depends on the type of buffer, and it increases with pH and temperature. The recommended assay conditions are: triethanolamine buffer 70 mmol/l, pH 7.6; *L*-alanine-4-nitroanilide concentrations depending on the species (see above); incubation temperature 25 °C.

Introduction

Measurements of "alanine aminopeptidase", α-aminoacylpeptide hydrolase (EC 3.4.11.2, microsomal), in urine provide a sensitive diagnostic indicator for renal diseases (1) and are particularly useful for the recognition and assessment of nephrotoxic side effects of drugs in both animal studies and human clinical investigations (2, 3). *L*-Alanine-4-nitroanilide and *L*-alanine-β-naphthylamide are the commonly used substrates for alanine aminopeptidase.

Kinetic studies showed that *L*-alanine-4-nitroanilide inhibits the alanine aminopeptidase reaction in human urine at concentrations exceeding about 0.8 mmol/l (3). The commonly used standard substrate concentration of 1.6 mmol/l inhibits alanine aminopeptidase by about 10 per cent, but the inhibition may be as

[1]) Report of the working group Clinical Chemistry of Laboratory Animals of the German Society of Clinical Chemistry.

high as 15 to 20% in some urines. It became necessary, therefore, to re-examine the assay conditions for alanine aminopeptidase in urine with *L*-alanine-4-nitroanilide. Since *L*-alanine-β-napthylamide also inhibits the reaction at concentrations exceeding about 0.7 mmol/l (l. c. (4), p. 37), this substrate was not considered as an alternative.

The aim of this multi-laboratory investigation was to optimize the recommended alanine aminopeptidase assay in urine, for both man and laboratory animals. Significant differences were found between species with regard to substrate inhibition, so that the optimal concentration of *L*-alanine-4-nitroanilide is different, depending on whether the assay is performed in the urine of man, rat, and dog. These substrate concentrations are close to the K_m values, which are different in each species.

Methods

Urine collection

Patient urines were randomly selected from the Clinical Chemistry Laboratory. Urines from mature Wistar rats and beagle dogs were collected from animals housed in metabolic cages in order to exclude contamination by food and faeces. Water was supplied ad libitum. During the collection period the urine was either cooled in ice or preserved by addition of sodium azide (10 µl of a 15 mmol/l solution per 10 ml of urine).

Preparation of urine

Urine was centrifuged for 10 min at 1000 *g* and the supernatant (= crude urine) used for gel filtration on ready to use columns, Sephadex G25, medium (Pharmacia PD 10). Crude urine (2.5 ml) was applied to the column and the eluate was discarded; after complete drainage, 4.0 ml NaCl 0.15 mol/l were added; the total eluate was collected for enzyme analysis; dilution factor = 1.6 (5).

Enzyme assay

The final concentrations in the incubation medium were:
L-alanine-4-nitroanilide 0.834 mmol/l, unless stated otherwise;
phosphate, Tris or triethanolamine buffer, 70 mmol/l, pH 7.6, unless stated otherwise;
sample volume fraction 0.2.
Urines with high alanine aminopeptidase catalytic activity concentration (see recommendation) were diluted with sodium chloride, 0.15 mol/l. Liberated 4-nitroaniline was measured at 405 nm in thermoregulated cuvettes at 25 °C.

Tab. 1. pH optima urine gel filtrates

Buffer	Tris			Triethanolamine		
	N	x[1]	Range	N	x[1]	Range
Man	8	7.8	7.6–7.9	10	7.9	7.8–8.1
Rat	21	8.0	7.7–8.4	20	8.0	7.7–8.4
Dog	5	7.7	7.6–8.1	5	7.8	7.6–8.2

[1] Median. Experiments with [S] = 0.834 mmol/l.

In the five laboratories the following analysers were used:
1 & 5, Flexigem, ENI, Inc.;
2, CentrifiChem 400, Union Carbide;
3, Cobas-Bio, Roche Analytical Systems;
4, Eppendorf Kinetic Enzyme Analyzer 5080, Eppendorf Gerätebau.

Results

pH optima

The pH dependence of the alanine aminopeptidase activity was measured between pH 7.0 and 9.0 in increments of 0.2. The pH optima with Tris and triethanolamine buffer are shown in table 1. There are slight differences between species and between buffers. Phosphate buffer was used between pH 7.0 and 8.0 without a maximum being reached. Exploratory experiments with rhesus monkeys and mini pigs also show pH optima near 8 with Tris and triethanolamine buffer. In figure 1, two crude and gel-filtered human urines are compared. The activity ratio gel filtrate/crude urine (GF/CR) is low in one urine and high in the other. Note the shift of the pH optimum to the alkaline side in the urine with the high gel filtrate/crude urine activity ratio. A high ratio means a relatively high concentration of inhibitors in the crude urine. Since amino acids were identified as naturally occurring alanine aminopeptidase inhibitors in urine (6), phenylalanine, which has the lowest $I_{0.5}$ (i. e. the inhibitor concentration which inhibits 50%) of the amino acids tested, was added to the gel filtrate of a human urine (fig. 2). This shifted the pH optimum to the alkaline side. In figure 3 the pH optima are compared between V_{max} and v at [S] = 0.834 mmol/l. Since K_m increases with increasing pH, the pH optimum is shifted to the acid side, depending on the substrate concentration.

Michaelis-Menten constants

The K_m values for *L*-alanine-4-nitroanilide were calculated by regression analysis from *Lineweaver-Burk* plots (1/v vs 1/[S]), with substrate concentrations ranging from 0.1668 to 16.68 mmol/l. In view of the inhibition of the alanine aminopeptidase activity by substrate concentrations exceeding ≈ 0.8 mmol/l with human urine and ≈ 0.3 – 0.5 mmol/l with animal urines, the K_m was calculated from the straight part of the plot at low substrate concentrations (6). See the discussion for details.

The results for gel filtrate of human urine are given in table 2. The lowest K_m values were obtained in triethanolamine buffer pH 7.6. The pH-dependent changes of K_m are also demonstrated in figure 3. In table 3 the K_m values are listed for rat and dog urine, and in both cases the lowest value was found in triethanolamine buffer.

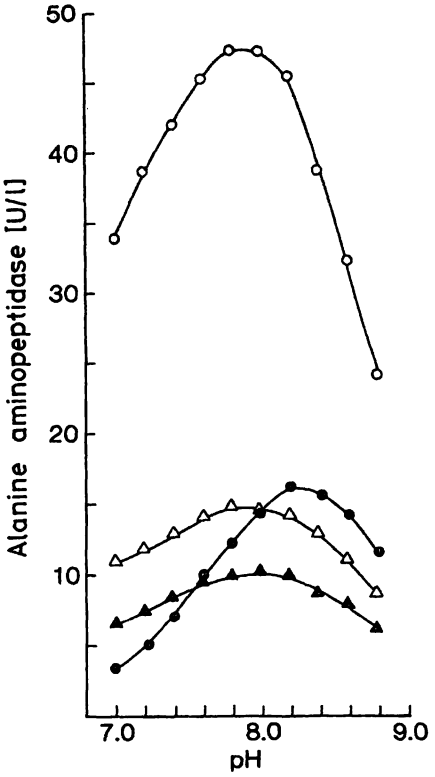

Fig. 1. pH dependence of alanine aminopeptidase in crude (CR) and gel-filtered (GF) human urine. One urine has a low activity ratio GF/CR (i. e. a low concentration of natural inhibitors in CR) (CR ▲, GF △), the other a high activity ratio GF/CR (i. e. a high concentration of natural inhibitors in CR) (CR ●, GF ○). Note the shift of the pH optimum to the alkaline side in CR with the high inhibitor concentration. Substrate concentration = 0.834 mmol/l, triethanolamine buffer.

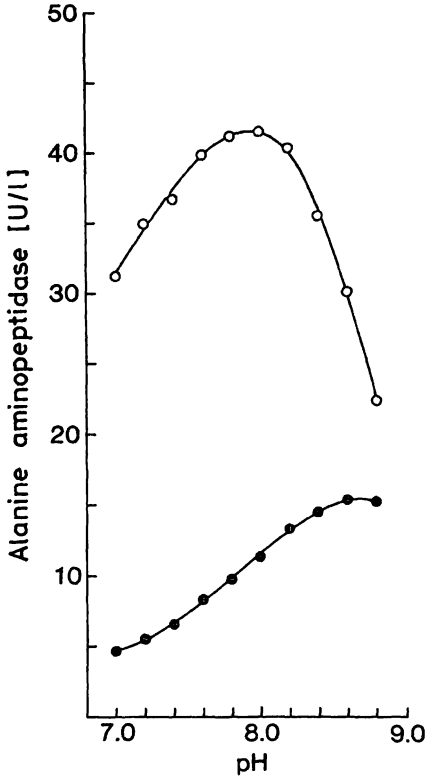

Fig. 2. pH dependence of alanine aminopeptidase in a gel filtrate of human urine, without (○) and with (●) the addition of 4 mmol/l phenylalanine, one of the identified natural inhibitors (6). Note the shift of the pH optimum to the alkaline side in the presence of the inhibitor. Substrate concentration 0.834 mmol/l, triethanolamine buffer.

Tab. 2. K_m in gel filtrate of human urine with various buffers

Buffer	Phosphate	Tris	Triethanolamine	
pH	7.6	7.6	7.6	7.8
N	28	5	15	5
x[1]	0.60	0.69	0.34	0.40
Range	0.42	0.41	0.18	0.25
	−0.88	−0.72	−0.39	−0.49

[1] Median. K_m in mmol/l.

Tab. 3. K_m in gel filtrate of rat and dog urine at pH 7.6 with various buffers

	Phosphate	Tris	Triethanol amine
Rat			
N	23	32	32
x[1]	0.52	0.41	0.17
Range	0.30	0.30	0.10
	−0.85	−0.72	−0.33
Dog			
N	4	4	4
x[1]	1.05	0.50	0.44
Range	0.76	0.36	0.40
	−1,09	−0.57	−0.45

[1] Median. K_m expressed in mmol/l.

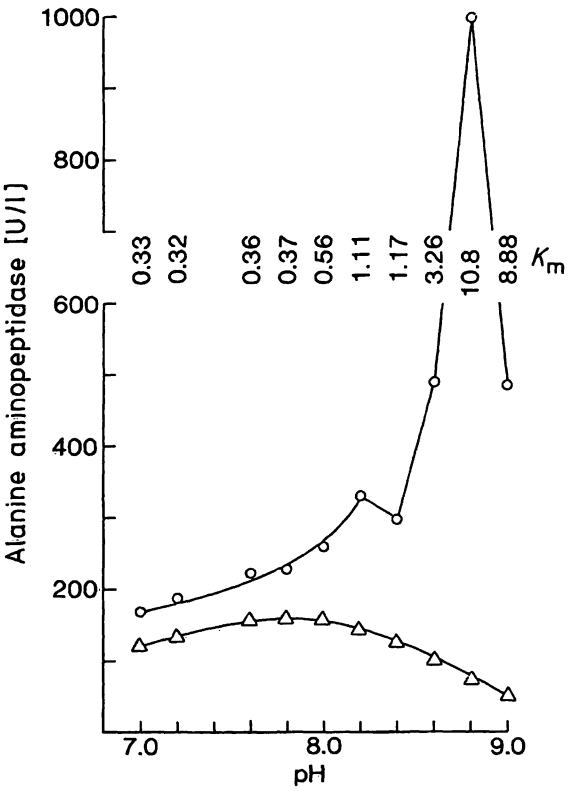

Fig. 3. pH dependence of alanine aminopeptidase in a gel filtrate of human urine. V_{max} ○, v at [S] = 0.834 mmol/l △, K_m inserted, triethanolamine buffer. The sharp pH-dependent peaks of V_{max} and K_m are consistent with an earlier report (9).

Stability of alanine aminopeptidase during storage

It is the experience of all participating laboratories, that crude urine can be stored at 4 °C without significant loss of activity for only 3–4 days. Freezing destroys the activity instantly. In contrast, the activity remains stable for at least three weeks in gel filtrates frozen at −20 °C. However, immediate preparation of gel filtrates may not always be practicable. Therefore, storage of crude urine with ethylene glycol was tested and found to be very satisfactory. The results are listed in table 4. No inhibition of alanine aminopeptidase activity by ethylene glycol was observed in experiments using up to 3 ml of the solvent added to 7 ml crude urine.

Tab. 4. Stability of alanine aminopeptidase in human and rat urine

Material	Temperature	N	Day 0	Day 7	Day 18
Human urine[1]	+ 4 °C	10	3.39 ± 2.18	3.41 ± 2.06	3.68 ± 2.01
Rat urine[1,2]	+ 4 °C	10	11.3 ± 7.40	12.4 ± 7.77	12.0 ± 7.55
Human urine[3]	−20 °C	10	3.35 ± 2.06	2.86 ± 1.77	3.40 ± 1.89
Rat urine[2,3]	−20 °C	9	8.12 ± 4.40	7.97 ± 4.83	8.38 ± 4.38

Expressed as U/l, mean ± s
[1] 8 ml urine + 2 ml ethylene glycol
[2] Refrigerated during collection (16 h)
[3] 7 ml urine + 3 ml ethylene glycol

Incubation temperature

The effect of the incubation temperature of the alanine aminopeptidase activity in human urine is shown in table 5. It should be noted that the K_m rises with the temperature. Consequently, the Q_{10} values are higher for V_{max} than for v at [S] = 0.834 mmol/l. The temperature effect, 37 °C vs 25 °C, in rat urine was measured at [S] = 834 mmol/l only. Urine was collected from six male and five female Wistar rats on two days, one week apart. There was no statistically significant difference between the two days and between sexes, and the results were therefore combined (N = 22 from 11 animals). The mean activity ratio 37 °C/25 °C was 2.93, s = 0.20. This represents a Q_{10} of 2.46, which is slightly higher than the values in table 5 with human urine.

Tab. 5. Effect of temperature on alanine aminopeptidase in human urine

Urine no.		25 °C	35 °C	Q_{10}
1	V_{max}	22.4	55.5	2.48
	K_m	0.26	0.40	
	v*	17.1	37.5	2.20
2	V_{max}	44.3	105	2.37
	K_m	0.28	0.40	
	v*	33.2	71	2.14
3	V_{max}	28.6	67.9	2.37
	K_m	0.39	0.57	
	v*	19.5	40.3	2.07

v* = activity at [S] = 0.834 mmol/l, triethanolamine buffer pH 7.6

Quality control

Imprecision was investigated for within enzyme assay runs, within and between gel filtration columns and enzyme assay, and day to day enzyme analyses. The very satisfactory results are given in tables 6, 7 and 8, respectively. The imprecision did not vary significantly between the different instruments used in the participating laboratories.

Tab. 6. Within-run imprecision of alanine aminopeptidase analyses in gel-filtered urine

	Rat		Human		
	v[a]	v[b]	v[c]	V_{max}[d,e]	K_m[d]
$\bar{x}$[e]	6.56	14.9	2.52	37.7	0.31
s	0.08	0.22	0.07	0.9	0.018
CV(%)	1.25	1.48	2.78	2.39	5.74
N	15	10	10	4	4
$\bar{x}$[e]	9.00	104	19.9		
s	0.18	1.75	0.41		
CV(%)	2.0	1.68	2.06		
N	15	10	10		
$\bar{x}$[e]	19.9				
s	0.33				
CV(%)	1.66				
N	15				

[a] [S] (mmol/l) = 1.67
[b] [S] = 0.83
[c] [S] = 0.04
[d] calculated from *Lineweaver-Burk* plot, K_m in mmol/l
[e] U/l

Discussion

The discussion of the results of this investigation and the recommendation will be preceded by a brief review of other methods that have been used and proposed for microsomal or particle-bound α-aminoacylpeptide hydrolases (EC 3.4.11.2), and by a description of the effect of identified inhibitors on the enzymes.

Tab. 7. Within column and between columns imprecision of gel filtration and alanine aminopeptidase assays in human urine

	Within column[a]	Between columns[b]
$\bar{x}$[c]	2.65	2.69
s	0.16	0.09
CV (%)	6.03	3.35

[a] Ten aliquots were passed through one column, and the gel was regenerated after each aliquot
[b] Aliquots were passed through ten individual columns
[c] U/l

Tab. 8. Day to day imprecision of alanine aminopeptidase analysis[a]

	Normosic	Kontrollogen-LP
$\bar{x}$[b]	17.0	22.35
s	0.39	0.41
CV (%)	2.29	1.83

[a] Commercial quality control preparations were analysed on ten consecutive working days
[b] U/l

The name alanine aminopeptidase was introduced in 1967 (7) for aminopeptidases which preferentially hydrolyse substrates with N-terminal alanine, such as *L*-alanine-β-naphthylamide (8) and *L*-alanine-4-nitroanilide (9). Substrates with N-terminal *L*-leucine are also hydrolysed, but at a slower rate, and *L*-leucine-β-naphthylamide (10) and *L*-leucine-4-nitroanilide (11) were recommended as substrates. Accordingly, the enzyme is called leucine aminopeptidase. The main advantages of the nitroanilides over the naphthylamides are the possibility of continuous recording of the release of 4-nitroaniline, and the easy adaptation of the assay to automated analysers.

Assay conditions, as used with both leucine and alanine nitroanilides, are summarized in table 9. The conditions vary considerably with regard to the choice of buffer, pH, concentrations of buffer and substrate, and assay temperature. Of these selected publications, two (14, 16) recommend standard assay conditions for human serum, and one (19) for human urine.

According to our results, the recommended concentrations of *L*-alanine-4-nitroanilide for both human serum (16) and urine (19) are too high, because the substrate inhibits the reaction.

Amino acids and ammonia were identified as natural inhibitors of alanine aminopeptidase in the urine of man and rat (6) and are quantitatively removed by gel filtration. In human urine they account for about one half of the removable inhibition potential.

In experiments with increasing concentrations of *L*-alanine-4-nitroanilide, any one of the four types of *Lineweaver-Burk* plots shown schematically in figure 4 may be encountered with human and animal urines. The ideal straight line (a) is only occasionally encountered and is found in crude urines with relatively high inhibitor concentrations, i.e. a relatively high value for the ratio: gel filtrate activity/crude urine activity (GF/CR). Urines with low inhibitor concentrations (low activity ratio GF/CR) give rise to plot (b), which is typical for substrate inhibition, and is invariably obtained with gel filtrates of human urine. Inhibitions begins at substrate concentrations > 0.8 mmol/l, and is always stronger in the gel filtrate than in the corresponding crude urine, i.e. after removal of the endogenous inhibitors from the urine. It was previously suggested that this effect may be caused

Tab. 9. Assay conditions for amino acid arylamidase measurements[a]

Author	Year	Enzyme source	Species	Buffer	Concentration mmol/l	pH	Substrate[b]	Concentration[c] mmol/l	T °C	Reference
Tuppy et al.	1962	kidney	pig	triethanolamine	100	8.0	LN	1.66	30	11[g]
Raab	1966	urine	rat	phosphate	48	7.2	LN	0.31	25	12
Wachsmuth et al.	1966	kidney	pig	phosphate	60	7.0	AN/LN	1.66	37	9
Jösch et al.	1967	urine	human	phosphate	47	7.2	LN	0.31	25	13
Bergmeyer et al.	1972	serum	human	Tris	50	7.5	LN	4.00	25	14[d,f]
Wiedmann et al.	1972	urine	human	phosphate	41	7.2	LN	0.78	25	15
Schlaeger	1973	serum	human	Tris	50	8.0	AN	2.00	25	16[d,f]
Lorentz et al.	1975	serum	human	Tris	250	7.8	AN	2.00	25	17[f]
Scherberich et al.	1976	urine	human	Tris	50	7.6	AN	1.66	25	18[e]
Jung et al.	1980	urine	human	Tris	50	7.8	AN	2.00	37	19[d]
Mondorf et al.	1983	urine	human	phosphate	70	7.6	AN	1.66	25	20
Berscheid et al.	1983	urine	rat	phosphate	70	7.6	AN	1.66	25	21
Hafkenscheid	1984	serum	human	Tris	250	7.8	AN	2.00	30	22
Mattenheimer et al.	1986	urine	human	phosphate	70	7.6	AN	1.66	25	6

[a] Compiled from selected publications; [b] LN = leucine-4-nitroanilide, AN = alanine-4-nitroanilide; [c] Concentration in assay mixture; [d] Recommendation for standardization; [e] Addition of 5 mmol/l $MgCl_2$; [f] Addition of 250 mmol/l NaCl; [g] Addition of 10 mmol/l $MgCl_2$

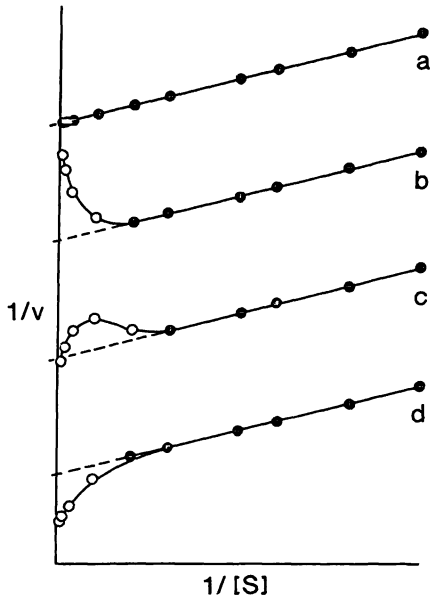

Fig. 4. Types of *Lineweaver-Burk* plots found with urine gel filtrates and *L*-alanine-4-nitroanilide as substrate: (a) ideal plot; (b) inhibition by substrate; (c) inhibition by substrate with a "hump" in the plot; observed regularly with rat urine and some, but not all dog urines; (d) presence of two distinct enzymes with different K_m and V_{max} values. See text for details.

by the removal of an unidentified activator from the crude urine by gel filtration (3). Experiments in which phenylalanine (one of the natural inhibitors in urine) was added to gel filtrate suggest, however, that the substrate inhibition may be diminished and abolished by a competition between substrate and inhibitor at the inhibitor binding site (6).

Gel filtrates of rat urine give rise to plot (c), which is characterized by a "hump". In the majority of rat urines, the ascending limb of the hump begins at a substrate concentration of > 0.3 mmol/l, in others at [S] > 0.5 mmol/l. Variations occur in the height (maximum inhibition) of the hump, even in urines collected on consecutive days from the same animal. These changes appear to follow an approximate six day cycle (*Grötsch*, unpublished). The shape of the plot may be caused by the existence of more than one alanine aminopeptidase (see below). Only occasionally does one encounter plot (b) with a rat urine.

The few dog urines that were investigated gave rise to plot (b); in three gel filtrates substrate inhibition began at [S] > 0.8 mmol/l and in one gel filtrate at [S] > 0.5 mmol/l.

Plot (d) signifies the presence of two distinct enzymes with different K_m values and catalytic concentrations, and it is occasionally seen in crude urine with high endogenous inhibitor concentrations. This type of plot is obtained by adding an inhibitor, e. g. phenylalanine, to gel filtrate (6). Similar results were recently obtained with rat urine (*Grötsch*, unpublished); with increasing amounts of phenylalanine in the reaction mixture the hump first disappears, and the plot than inflects downward.

In figure 5 the *Lineweaver-Burk* plots are compared for the gel-filtrates of human and rat urine, and for different buffers. Since the *Lineweaver-Burk* plots with gel filtrates deviate from linearity at higher substrate concentrations, the estimation of K_m is restricted to the extrapolation from the straight part at low substrate concentrations. Although fairly reliable estimates of K_m may be expected from such extrapolations, the situation is complicated by the presence of two alanine aminopeptidases (E_1 and E_2) in urine, as has been demonstrated in inhibition experiments with phenylalanine (6). The K_m of E_1 is low ($\approx$ 0.1 mmol/l) and that of E_2 is high ($\approx$ 2 mmol/l). Consequently, extrapolation of the straight segment of the *Lineweaver-Burk* plot at low substrate concentrations yields V_{max} and K_m mainly for E_1; but as the activity ratio E_1/E_2 varies from urine to urine, so does the contribution of E_2 to the activity at low substrate concentrations. This may explain the relatively wide range of K_m values which was found (tabs. 2, 3). The imprecision of the V_{max} and K_m determination per se is low (tab. 6).

Considering the present study and the need for a recommended procedure for the measurement of alanine aminopeptidase in urine, the following results should be recalled:

(1) The inhibition of alanine aminopeptidase activity in human and animal urine by alanine-4-nitroanilide in excess of about 0.8 (man), 0.3 (rat), and 0.5 mmol/l (dog), is very disadvantageous, because the apparent K_m values lie in the range from 0.2 to 1 mmol/l;

(2) the pH optima differ only slightly between Tris and triethanolamine buffer (tab. 1);

(3) K_m increases with increasing pH (tab. 2 and fig. 3), and the lowest value was found with triethanolamine buffer (tabs. 2 and 3);

(4) K_m also increases with increasing temperature (tab. 5).

Because of the substrate inhibition, assays cannot be done at substrate saturation. Therefore, triethanolamine was selected, the buffer in which the lowest K_m was found. Since K_m is both pH and temperature dependent, pH 7.6 and 25 °C are recommended.

The recommendation of 25 °C as incubation temperature is not in line with the increasing acceptance of 37 °C for enzyme activity assays. The authors strongly

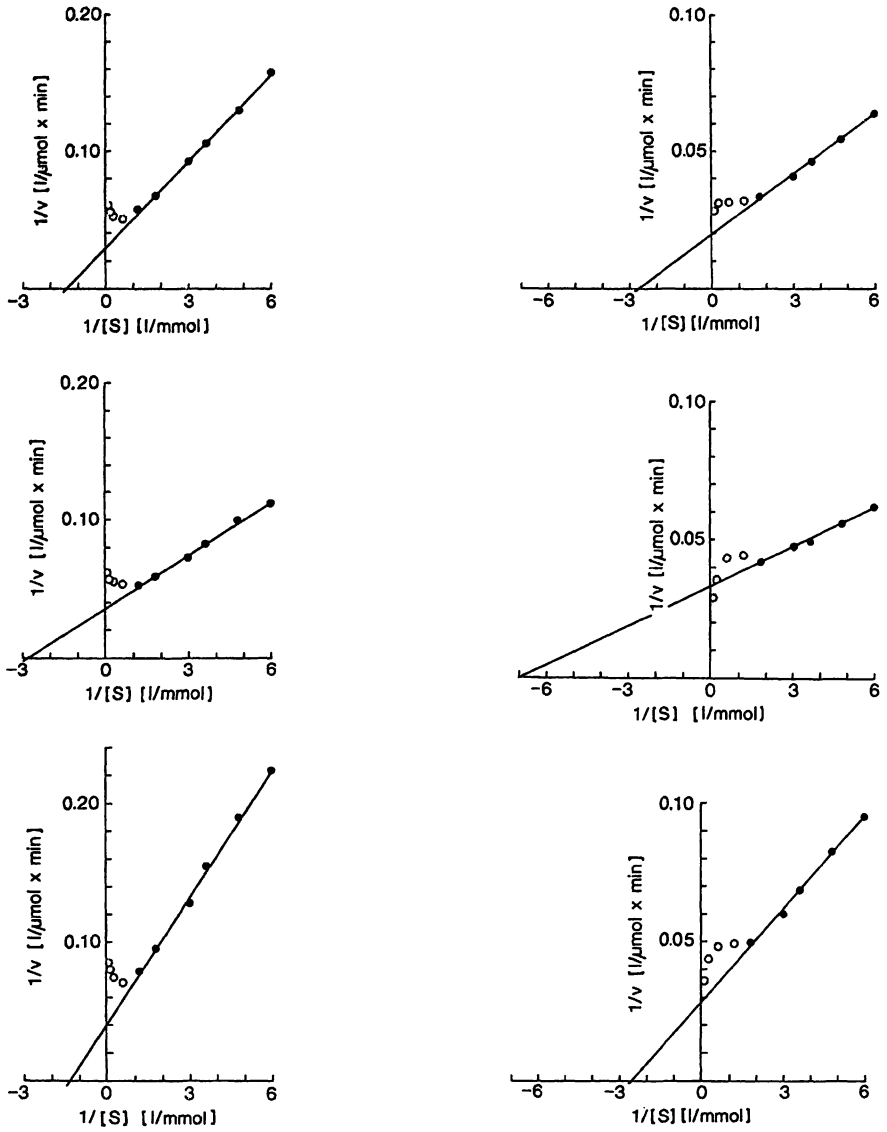

Fig. 5. Determination of V_{max} and K_m in human (left) and rat (right) urine with Tris (top), triethanolamine (middle), and phosphate buffer (bottom), all at pH 7.6 and 70 mmol/l final concentration. The same gel filtrate was used with all three buffers. Deviation from the straight line begins in human urine at [S] > 0.834 mmol/l and in rat urine at [S] > 0.556 mmol/l. The first open circle (from the right) deviates over 15% with human urine and over 10% with rat urine from the calculated regression lines (filled circles).
* Recommended substrate concentration for human and rat urine, respectively.

	K_m	V_{max}		v at [S] = 0.8 mmol/l*			K_m	V_{max}		v at [S] = 0.3 mmol/l*		
		U/l	relative	U/l	relative	% of V_{max}		U/l	relative	U/l	relative	% of V_{max}
Tris	0.70	33.1	1.0	17.6	1.0	53	0.36	50.3	1.0	22.9	1.0	46
Triethanolamine	0.35	27.5	0.83	19.1	1.09	69	0.14	30.4	0.60	20.7	0.90	68
Phosphate	0.78	24.5	0.74	12.4	1.70	51	0.39	34.9	0.69	15.2	0.66	43

believe, however, that the experimental data of this investigation justify the exception.

The distinct peaks of K_m and pH optimum in figure 3, including the shoulder around pH 8, were also found in other human urines and in an extract from human kidney cortex. The results are consistent with an earlier investigation with a purified aminopeptidase from pig kidney (9).

The difference in the onset of the substrate inhibition between species, [S] > 0.8 mmol/l in human urine, [S] > 0.3 — > 0.5 mmol/l in rat urine, and [S] > 0.5 — > 0.8 mmol/l in dog urine, does not permit the recommendation of an alanine aminopeptidase assay equally suitable for both human and animal urine. The applicable substrate concentration of 0.8 mmol/l in human urine is only about 2.5 times higher than the median K_m value of 0.34 in triethanolamine buffer.

It would therefore not be advisable to lower the substrate concentration just for the purpose of conforming with conditions in animal urines. A substrate concentration of 0.3 mmol/l for the rat, and of 0.5 mmol/l for the dog must be recommended, although these concentrations are about equal to the respective K_m values.

The results in figure 5 also reveal that not only K_m but also the velocity of the enzyme reaction depends on the buffer. In human and rat urine the highest V_{max} values were obtained with Tris buffer. However, because of the differences of K_m between buffers, the velocities at [S] = 0.8 mmol/l in man, and [S] = 0.3 mmol/l in the rat with triethanolamine buffer are close to the velocities in Tris buffer. The species differences, with respect to the relative activities in different buffers, should also be noted.

The effect that sodium chloride may have on the activity of alanine aminopeptidase with *L*-alanine-4-nitroanilide as substrate appears to be controversial. With human serum and a substrate concentration of 2 mmol/l, an activity increase of 10 to 20 percent with 250 mmol/l sodium chloride was found in one study (17) but not in another (16); the K_m was not affected (17). The activity was only insignificantly increased in a gel filtrate of human urine under comparable assay conditions (19). In the present investigation Sephadex columns are used equilibrated with 150 mmol/l sodium chloride, and urine as eluted with the same solution. The gel filtrate of rat urine was found to contain on average (in mmol/l) 135 sodium and 128 chloride ions, and < 1 potassium, phosphate and bicarbonate ions. Gel filtrate of human urine contained on average 120 mmol/l chloride ions; other ions were not determined. With the recommended sample volume fraction of 0.2, the sodium chloride concentration in the assay mixture amounts to about 25 mmol/l. Sodium chloride added to the assay mixture in a final concentration of 250 mmol/l increased the V_{max} of alanine aminopeptidase in a gel filtrate of human urine by 20 percent, and the K_m from 0.3 to 0.4 mmol/l. Consequently, the positive effect of sodium chloride on the activity was only about 7 percent at the recommended substrate concentration of 0.8 mmol/l. Since K_m had to be kept as low as possible in the recommended method, addition of sodium chloride to the assay was not considered.

The results of urine preservation by storage of gel filtrates at −20 °C are in agreement with earlier reports (19, 23). Crude urine mixed with ethylene glycol or glycerol and stored at −20 °C also provides a stable quality control material (24).

Intentionally, this recommendation does not comment on the pros and cons of timed or untimed urine collection, the problem of diuresis-dependency, and the appropriate basis of reference (e. g. urinary creatinine). This problem applies to most macromolecular analytes in urine and is still under discussion (25, 26).

Recommendation

Please note in the following recommendation, that storage and preparation for enzyme analysis are identical for human and animal urine. In the alanine aminopeptidase assay, however, the substrate concentrations differ and are specific for man, rat and dog.

Urine collection

Human urine should be collected without the addition of a preservative. During collection periods exceeding about 3 h the collection bottle should be kept in a refrigerator.

Animal urine should be collected from animals housed individually in metabolic cages. During the collection period the urine should be cooled in ice or preserved by the addition of sodium azide (10 µl of a 15 mmol/l solution per 10 ml of urine).

Rats excrete 5−10 ml of urine per 24 h. However, after placing a rat in a metabolic cage, the urine volume is often reduced to less than 1 ml during the first few days. Therefore, urine collection should begin 4−5 days after caging, provided the experimental protocol permits this delay.

Urine storage

Crude urine

Centrifuge the urine for 10 min at 1000 *g*. Procedure A: to 8 ml of the supernatant add 2 ml of ethylene glycol (E. Merck, Darmstadt, art. no. 9621, analytical grade). Store at 4 °C. Procedure B: Alternatively, to 7 ml of the supernatant add 3 ml ethylene glycol. Store at −20 °C. Scale down either procedure for smaller urine volumes. The dilution factor of 1.25 (procedure A) or 1.43 (procedure B) must be considered in the final calculation of the enzyme activity.

Gel filtrate

Gel filtrate may be stored at −20 °C without any additive.

Urine preparation

Allow stored urine to reach room temperature. Centrifuge fresh urine as described above. For gel filtration on "ready to use" columns of Sephadex G25 medium (Pharmacia PD 10), apply 2.50 ml supernatant, drain completely, and discard the eluate. Add 4.0 ml 0.15 mol/l NaCl. The total eluate is collected and used for the enzyme analysis.

Enzyme assay for human urine

Prepare incubation mixture in cuvettes (d = 1.0 cm) and read or record absorbance changes in a spectrophotometer or spectrum-line photometer at 405 nm. Incubation temperature 25 °C. Incubation volume 500 µl.

Incubation mixture:	µl	final concentration
Triethanolamine buffer 100 mmol/l, pH 7.6	350	70 mmol/l
Sample	100	
L-Alanine-4-nitroanilide, 8.0 mmol/l	50	0.8 mmol/l

Read or record absorbance for 3 min (longer with low activities).

Calculation

Alanine aminopeptidase U/l =

$$\frac{\Delta A}{\Delta t} \times \frac{10^5}{990} \times \frac{500}{100} = \frac{\Delta A}{\Delta t} \times 505 \; (\times \text{ dilution factor})$$

where 990 m^2/mol is the molar lineic absorbance of 4-nitroaniline at 405 nm.

Enzyme assay for animal urines

For animal urines lower substrate concentrations must be recommended, because of the onset of substrate inhibition at concentrations lower than in human urine. Otherwise the assay remains the same as with human urine.

The recommended final concentrations of *L*-alanine-4-nitroanilide are 0.3 mmol/l for rat, and 0.5 mmol/l for dog urine.

Notes

Because the recommended substrate concentrations are low with reference to the K_m values, special care must be taken not to exceed about 5 percent hydrolysis of the substrate.

Examples, assuming a 3 min incubation period

Human urine: Median value of K_m = 0.34, and [S] = 0.8 mmol/l. Total ΔA should not exceed 0.400. ΔA/3 min = 0.400, ΔA/min = 0.133 or 0.133 × 505 × 1.6 = 107 U/l.

Rat urine: Median value of K_m = 0.19, and [S] = 0.3 mmol/l. Total ΔA should not exceed 0.150. ΔA/3 min = 0.150, ΔA/min = 0.050 or 0.050 × 505 × 1.6 = 40 U/l.

Dog urine: Median value of K_m = 0.44, and [S] = 0.5 mmol/l. Total ΔA should not exceed 0.250. ΔA/3 min = 0.250, ΔA/min = 0.083 or 0.083 × 505 × 1.6 = 67 U/l.

For urines mixed with ethylene glycol for storage, the appropriate additional dilution factor of 1.25 (procedure A) or 1.43 (procedure B, see urine storage) must be applied. If necessary, the gel filtrate should be diluted with saline.

Caution is also advised in the interpretation of results from experimental studies with animals and diagnostic applications with patients. In addition to the already disadvantageous ratio of substrate concentration to K_m, the established K_m values vary over a relatively wide range (the dog appears to be an exception, but urines were studied from only 4 animals, compared with 15 human urines and 32 from the rat).

Tab. 10. Activity calculated as fraction (F) of V_{max} at the recommended substrate concentrations

Species	[S] mmol/l	K_m mmol/l		F at [S] absolute	F at [S] relative	[S]−5% mmol/l	F at [S]−5%	F at [S]−5% / F at [S]
Human	0.8	median	0.34	0.70	1.0	0.76	0.69	0.99
		low	0.18	0.82	1.17		0.81	0.99
		high	0.39	0.67	0.96		0.66	0.99
Rat	0.3	median	0.17	0.64	1.0	0.285	0.63	0.98
		low	0.10	0.75	1.17		0.74	0.99
		high	0.33	0.48	0.75		0.46	0.96
Dog	0.5	median	0.44	0.53	1.0	0.475	0.52	0.98
		low	0.40	0.56	1.06		0.54	0.96
		high	0.45	0.53	1.0		0.51	0.96

Table 10 lists the alanine aminopeptidase activities as fractions of V_{max} at the recommended substrate concentrations, calculated with the intermediate, lowest, and highest K_m for each species (K_m values from tabs. 2 and 3). Small activity changes, i. e. 17% in human and rat urine, may lie, therefore, within a normal biological variation. The last column shows that the decrease of activity after hydrolysis of five per cent of substrate is within acceptable limits.

Acknowledgement

For skillful technical assistance and support we are indebted to Ms. *Henny DeBruin*, Ms. *Edith Dichtl*, Ms. *Birgit Heiser*, Ms. *Christine Hawly*, and Mr. *Heinz Klieber*.

References

1. Burchard, U., Haschen, R. J. & Krosch, H. (1979) In: Diagnostic Significance of Enzymes and Proteins in Urine (Dubach, U. C. & Schmidt, U., eds.) Hans Huber, Bern, Stuttgart, Vienna, pp. 106–112.
2. Mondorf, A. W., Hendus, J., Breier, J., Maske, L., Bindzi, C., Scherberich, J. E. & Schoppe, W. (1979) In: Diagnostic Significance of Enzymes and Proteins in Urine (Dubach, U. C. & Schmidt, U., eds.) Hans Huber, Bern, Stuttgart, Vienna, pp. 192–200.
3. Mattenheimer, H. (1986) In: Clinical Enzymology Symposia 5, Proceedings of the 11th International Symposium on Clinical Enzymology, Rome, 1984 (Burlina, A. & Galzigna, L., eds.) Piccin, Padua, pp. 47–62.
4. Haschen, R. J. (ed.) (1972) Alanin-Aminopeptidasen, Biochemie und diagnostische Bedeutung; Wiss. Beitr. d. Martin-Luther-Universität Halle–Wittenberg (R17).
5. Grötsch, H. & Mattenheimer, H. (1983) In: Methods of Enzymatic Analysis, 3rd edn. (Bergmeyer, H. U. & Grassl, M., eds.) Vol. III, pp. 42–49, Verlag Chemie, Weinheim, Deerfield Bach Fl, Basel.
6. Mattenheimer, H., Frölke, W., Grötsch, H. & Simane, Z. (1986) Clin. Chim. Acta *160*, 129–135.
7. Rehfeld, N., Peters, J. E., Giesecke, H. & Haschen, R. J. (1967) Acta Biol. Med. Germ. *19*, 819–830.
8. Peters, J. E., Schneider, I. & Haschen, R. J. (1972) Clin. Chim. Acta *36*, 289–301.
9. Wachsmuth, E. D., Fritze, I. & Pfleiderer, G. (1966) Biochemistry *5*, 169–174.
10. Goldberg, J. A. & Rutenberg, A. M. (1958) Cancer *11*, 283–291.
11. Tuppy, H., Wiesbauer, U. & Wintersberger, E. (1966) Hoppe-Seylers Z. Physiol. Chem. *329*, 278–288.
12. Raab, W. (1966) Z. Klin. Chem. *4*, 56–58.
13. Jösch, B. & Dubach, U. C. (1967) Z. Klin. Chem. Klin. Biochem. *5*, 59–63.
14. Bergmeyer, H. U. et al. (Kommission für Enzymdiagnostik und Standardisierung) (1972) Z. Klin. Chem. Klin. Biochem. *11*, 192.
15. Wiedmann, M., Heidbreder, E., Hennemann, H. & Heidland, A. (1972) Med. Welt *23* (N. F.), 686–690.
16. Schlaeger, R. (1973) Z. Klin. Chem. Klin. Biochem. *11*, 326–328.
17. Lorentz, K., Koch, C. D., Flatter, B. & Molz, J. (1975) Z. Klin. Chem. Klin. Biochem. *13*, 49–52.
18. Scherberich, J. E., Mondorf, A. W. & Jost, R. (1976) Klin. Wochenschr. *54*, 1193–1195.
19. Jung, K. & Scholz, K. (1980) Clin. Chem. *26*, 1251–1254.
20. Mondorf, A. W., Heynold, F. T., Scherberich, J. E. (1983) Infection *11*, (Suppl. 1) S57–S62.
21. Berscheid, G., Grötsch, H., Hropot, M., Klaus, E. & Mattenheimer, H. (1983) J. Clin. Chem. Clin. Biochem. *21*, 799–804.
22. Hafkenscheid, J. C. M. (1984) In: Methods of Enzymatic Analysis, 3rd edn. (Bergmeyer, H. U. & Grassl, M., eds.) Vol. 5, pp. 11–15, Verlag Chemie, Deerfield Beach FL, Basel.
23. Philippon, Cl., Sansot, J. L., Catteneo, A., Prevot, D. & Manuel, Y. (1986) Clin. Chem. *32*, 699.
24. Maruhn, D., Wehling, K. & Metz, U. (1986) Clin. Chim. Acta *160*, 119–122.
25. Jung, K., Schulze, G. & Reinholdt, C. (1986) Clin. Chem. *32*, 529–532.
26. Ginsberg, J. M., Chang, B. S., Matarese, R. A. & Garella, S. (1983) New Engl. J. Med. *309*, 1543–1546.

Prof. Dr. med. Hermann Mattenheimer
Rush-Presbyterian-St. Luke's Medical Center
1653 West Congress Parkway
Chicago, IL, 60612, USA